32

HLA DNA TYPING

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The HLA region, located on the short arm of chromosome 6, encodes a set of highly polymorphic integral membrane proteins that bind antigen peptide fragments. This complex of HLA proteins and antigen peptide is recognized by the T-cell receptor, leading to activation of the T lymphocyte and the initiation of a specific immune response. The HLA class II loci (e.g., HLA-DR, -DQ, and -DP) encode an α and a β glycopeptide chain; this cell surface heterodimer presents antigen to $CD4^+$ T lymphocytes. The HLA class I loci, (e.g., HLA-A, -B, and -C) encode a glycopeptide chain that, associated with β-2 microglobulin, binds antigen and is recognized by $CD8^+$ T lymphocytes (reviewed in Kappes and Strominger 1988).

The detection of genetic variation in the HLA region (or "HLA typing") is useful in tissue typing for transplantation to minimize graft rejection by selecting "HLA-matched" donor and recipient pairs. HLA typing has also proved highly informative in the analysis of genetic susceptibility to autoimmune diseases, since specific alleles at certain HLA loci have been associated with particular diseases (e.g., HLA-DR3 and DR4 with insulin-dependent diabetes mellitus). The degree of polymorphism at these loci has also made HLA

PCR Protocols: A Guide to Methods and Applications

EXHIBIT G

typing valuable for individual identification in forensic analysis and paternity determination.

Traditionally, HLA typing has been carried out with serologic reagents or, in the case of the class II loci, by the mixed-lymphocyte culture (MLC) in which T lymphocytes from one sample will proliferate in response to different or "nonmatching" HLA class II gene products on the cell surface of cells from the other sample (reviewed in Bodmer 1984). More recently, with the availability of cloned HLA cDNA and genomic hybridization probes, HLA typing at the DNA level could be carried out by restriction fragment length polymorphism (RFLP). DNA typing offers a number of advantages over immunologic typing; these have been reviewed elsewhere (Erlich *et al.* 1986). RFLP analysis is based on the presence or absence of polymorphic restriction sites located primarily in noncoding regions that are in linkage disequilibrium (nonrandom association) with allelic variation in coding sequences. Until recently, the direct analysis of coding sequence polymorphism has been difficult. However, the enzymatic amplification of specific DNA sequences by PCR has provided a new approach to genetic typing.

PCR/Oligonucleotide Probe Typing

The capacity of the PCR to amplify a specific segment of genomic DNA has made it an invaluable tool in the study of polymorphism and evolution, as well as in the analysis of genetic susceptibility to disease. In all of these areas, a particular gene must be examined in a variety of individuals; either within a species, in different closely related species, or in patient and in healthy control populations. Here, we will focus on the use of PCR to perform HLA class II DNA typing and will use the analysis of allelic diversity at the HLA-DQα (now designated the DQA1) locus as an illustrative example. The polymorphism of class II genes is localized primarily to the NH_2 terminal outer domain encoded by the second exon. Using PCR primers to conserved regions, we have amplified and sequenced the second exon of these class II loci from many different individuals, revealing a remarkable degree of allelic diversity (Fig. 1). For some of these loci (e.g., DRβI), it is likely additional alleles will be revealed as samples from a variety of ethnic groups are analyzed. The sequences of the amplification primers used are shown in Table 1. These sequences

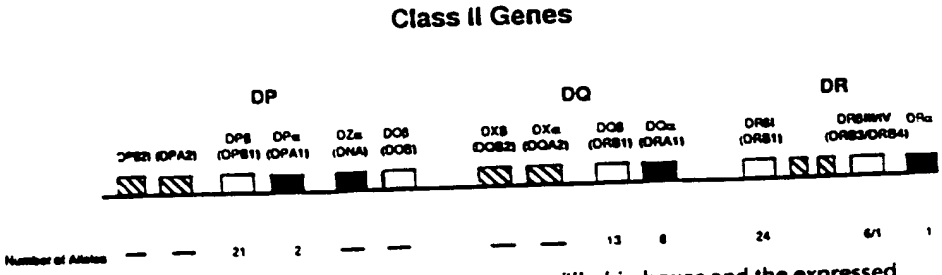


Figure 1 The expressed class II α loci are shown as filled-in boxes and the expressed β chain loci are shown as open boxes. Nonexpressed genes are represented by hatched boxes. The new nomenclature for these loci is shown in parentheses. In the DR region, some haplotypes (e.g., DRw8 and DR1) have only one expressed β locus. The number of alleles identified by us thus far are shown below the locus.

were determined either by M13 cloning of the DNA amplified with "linker-primers" (Scharf *et al.* 1986; see Chapter 4) followed by chain termination sequencing of the purified single-stranded phage DNA or by direct sequencing using the asymmetric primer method (Gyllensten and Erlich 1988; see Chapter 10) to generate single strands from the PCR.

Having determined the extent of allelic diversity by sequence analysis, one can then detect the presence of specific alleles in a PCR-amplified sample by dot blot hybridization with labeled oligonucleotide probes (Saiki *et al.* 1986). We have used this procedure with either ^{32}P, biotin, or horseradish peroxidase-labeled oligonucleotide probes for HLA-DQα, HLA-DQβ, HLA-DRβ, and HLA-DPβ typing (Bugawan *et al.* 1988; Horn *et al.* 1988; Scharf *et al.* 1988; Bugawan *et al.* 1989). This type of simple, rapid, and precise test is critical in typing the large number of patient and control samples necessary for the analysis of genetic susceptibility to disease. Unlike immunologic or RFLP approaches to HLA typing, the sequence based PCR/oligonucleotide probe analysis reveals not only that two alleles are different but *how* they differ, allowing the identification of individual polymorphic residues that are critical to disease susceptibility, graft rejection, T-cell recognition, antigen presentation, or whatever biological function is being examined.

The dot blot typing approach involves the PCR amplification of a specific region (i.e., the second exon of the DQα locus) and the subsequent immobilization of the amplified DNA to replicate filters (nylon membranes). The protein sequence alignment for the DQα alleles is shown in Fig. 2, and the nucleotide sequence alignment and the location of the oligonucleotide probes are shown in Fig. 3. Each

Table 1

Sequence of Oligonucleotide PCR Primers for HLA Class II Gene Amplification

HLA gene	Name	Sequence 5′ to 3′	PRC amplification conditions		
			Denature	Anneal	Extend
DRβ	GH46	CCGGATCCTTCGTGTCCCCACAGCACG	96°C	55°C	72°C
	GH50	CTCCCCAACCCCGTAGTTGTGTCTGCA			
DQα	GH26	GTGCTGCAGGTGTAAACTTGTACCAG	96°C	65°C	≥65°C
	GH27	CACGGATCCGGTAGCAGCGGTAGAGTTG			
DQβ	GH28	CTCGGATCCGCATGTGCTACTTCACCAACG	96°C	55°C	72°C
	GH29	GAGCTGCAGGTAGTTGTGTCTGCACAC			
DPα	GH98	CGCGGATCCTGTGTCAACTTATGCCGC	96°C	55°C	72°C
	GH99	GTGGCTGCAGTGTGGTTGGAACGC			
DPβ	UG21	CGGATCCGGCCCAAAGCCCTCACTC	96°C	65°C	≥65°C
	UG19	GCTGCAGGAGAGTGGCGCCTCCGCTCAT			

The denaturation, annealing, and extension times are 30 seconds, and ramping from 96° to 55° to 72°C is programmed in the PECI ThermoCycler for 1 sec. In some cases (DQα and DPβ), the temperature profile is a 2-step cycle from 65°C to 96°C. With these conditions, the DQα primers (GH26 and GH27) and the DQβ primers (GH28 and GH29) co-amplify to a limited extent the homologous sequence from the linked but nonexpressed loci DXα and DXβ. The DRβ primers GH46 and GH50 amplify *all* DRβ loci not simply the DRβ1 locus.

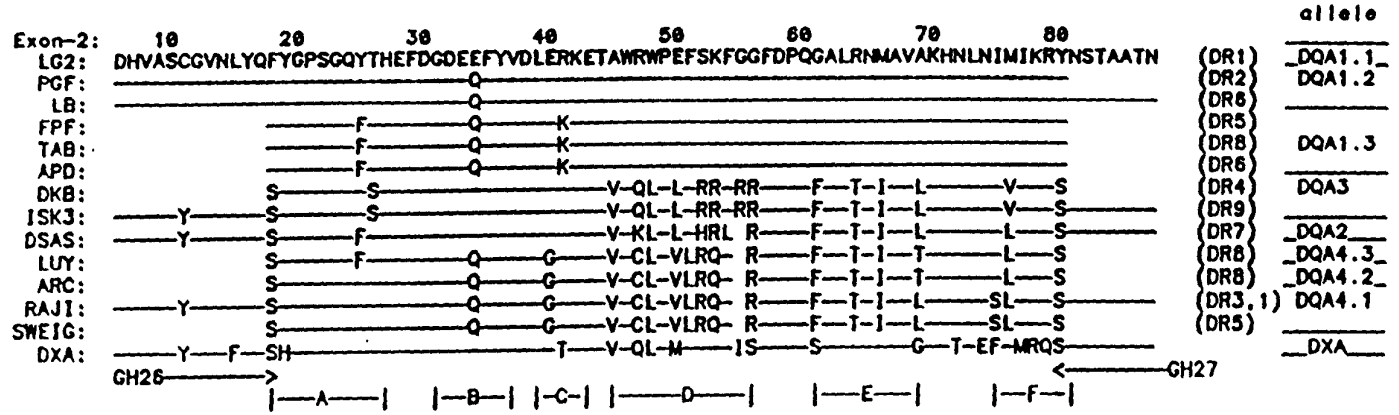
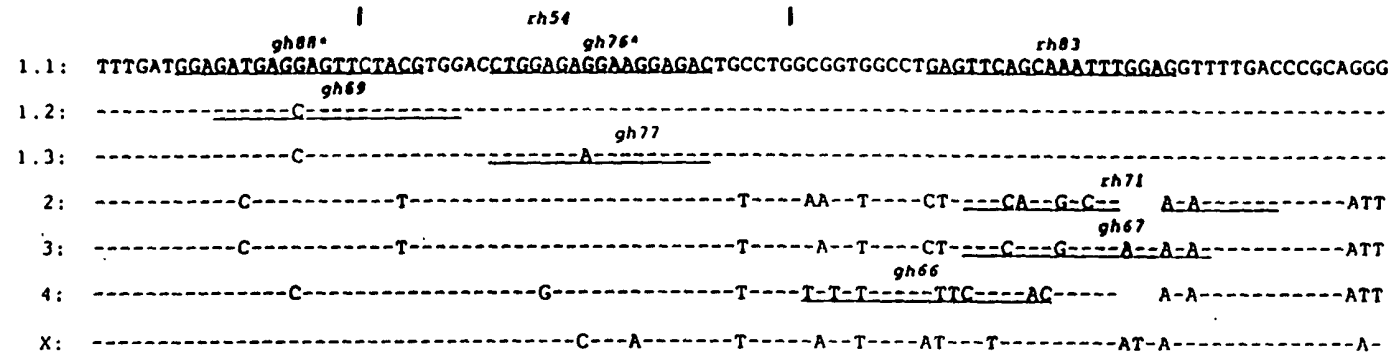


Figure 2 HLA-DQα protein sequences. The DQα allele designations are shown on the right. The names of representative cells are shown to the left, and their DR serotypes are shown to the right of the sequences. Polymorphic segments corresponding to specific oligonucleotide probes are designated A through F.



* = probe sequence is other strand

probe	specificity	length	sequence, 5' to 3'
RH54	all	33-MER	CTACGTGGACCTGGAGAGGAAGGAGACTGCCTG
RH83	1	19-MER	GAGTTCAGCAAATTTGGAG
GH88	1.1	19-MER	CGTAGAACTCCTCATCTCC
GH76	all but 1.3	17-MER	GTCTCCTTCCTCTCCAG
GH89	1.2, 1.3, 4	19-MER	GATGAGCAGTTCTACGTGG
GH77	1.3	17-MER	CTGGAGAAGAAGGAGAC
RH71	2	21-MER	TTCCACAGACTTAGATTTGAC
GH67	3	19-MER	TTCCGCAGATTTAGAAGAT
GH66	4	19-MER	TGTTTGCCTGTTCTCAGAC

Figure 3 HLA-DQα DNA sequence. The DQα DNA sequence and location of allele-specific oligonucleotide probe are shown. The probe name, sequence, and its specificity are shown on the bottom of the figure.

filter is then hybridized with a labeled oligonucleotide probe, and the bound probe is detected by the enzymatic conversion of a colorless soluble substrate to a colored precipitate. A detailed description of the protocol for HLA-DQα typing is given below. For a locus with *n* alleles, each amplified sample must be immobilized on *n* membranes and each membrane hybridized to one of *n* labeled probes. Thus, the procedural complexity of this approach is a function of the number of oligonucleotide probes required for complete genetic analysis. To address this problem, we have recently developed a "reverse dot-blot" procedure in which the oligonucleotide probe is immobilized on a membrane and hybridized to a labeled PCR product (Saiki *et al.* 1989). In this method, a panel of oligonucleotide probes is tailed with poly(dT) using terminal transferase and UV-cross-linked to a nylon membrane. The PCR product, labeled during amplification by using biotinylated primers, is then hybridized to the immobilized array of oligonucleotide probes. The presence of the specifically bound PCR product is detected using a streptavidin-horseradish peroxidase conjugate. Both the dot blot and the reverse dot blot method represent rapid and precise approaches for typing HLA class II polymorphism.

Protocols

DQα-PCR Amplification

1. Prepare PCR mixture containing the following components (see Chapter 1).

DNA	10 pg to 1 μg
10× *Taq* salts	10 μl (500 m*M* KCl, 15 m*M* $MgCl_2$,
100 m*M* dNTPs (25 m*M* each)	0.7 μl
10 μ*M* GH26	2.5 μl
10 μ*M* GH27	2.5 μl

   5 units/μl *Taq* Polymerase 0.6 μl
   Bring up to 100 μl with glass-distilled water.
2. Include a positive (DNA that is known to successfully amplify for DQα) and negative (no DNA) control to check for PCR efficiency, specific oligonucleotide probe hybridization, and contamination.

3. Mix reaction by vortexing lightly.
4. Amplify for 25 cycles (more if less than 0.1 μg starting DNA) in Perkin-Elmer Cetus Thermal cycler. Denature at 94°C for 30 seconds and anneal and extend at 65°C for 30 seconds.
5. Load 3 μl of PCR into 3% NuSieve plus 1% agarose gel to monitor amplification efficiency.

Preparation of Dot Blots

Prepare four replicate dot blots by spotting 5 μl of denatured PCR product per dot onto Genatran (or ZetaProbe) membrane. For detailed protocol for dot blot preparation, see Chapter 15. The immobilized amplification products are each hybridized with one of four DQα-ASO probes to determine the four allelic major types (DQA1, DQA2, DQA3, DQA4). If subtyping is necessary, membranes can be stripped and rehybridized with DQA1 subtyping probes, or additional membrane can be prepared.

Allele-Specific Oligonucleotide Probe Hybridization

1. Pre-wet the dot blot membranes in water or 2× SSPE.
2. Prehybridize the membranes in Seal-a-Meal bags containing just enough hybridization solution (5× SSPE, 5× Denhardt's and 0.5% Triton X-100) to cover the membrane (for a full-size dot blot use 8 ml solution). Incubate the membranes for 5 to 10 minutes at 55°C.
3. Add the HRP-labeled Allele Specific Oligonucleotide probe at 1 pmol/ml hybridization solution into the bag in the liquid. Hybridize for 30 minutes at 55°C.
4. Wash the membranes in 2× SSPE, 0.1% Triton X-100 for 5 minutes at 55°C.

Detection

The presence of the probes is detected by using a colorless soluble substrate that is converted by HRP, in presence of H_2O_2, to a colored precipitate. The detection procedure was carried out at room temperature with moderate shaking (see Chapter 15).

1. Incubate the membranes for 5 minutes with Buffer B [137 m*M* NaCL, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.0 m*M* Na_2HPO_4 (pH 7.4), 5% (v/v) Triton X-100, 1 *M* Urea, and 1% Dextran Sulfate].

2. Wash the membranes for 5 minutes in Buffer C [100 m*M* Sodium Citrate (pH 5)].
3. Incubate the membranes for 10 minutes in Buffer C plus 0.1 mg/ml of TMB (3,3' 5,5'- tetramethlybenzidine in 100% Ethanol) under light exclusion.
4. Add 0.0015% hydrogen peroxide for blue positive signal to appear, which usually takes 1 to 5 minutes.
5. Stop the color development by washing the membranes in Buffer C or water for 20 minutes. If filter background is high, additional washing may be necessary. The membranes can be stored in Buffer C in the absence of light at 4°C with little fading of signals for at least 2 months.

Rehybridization

If subtyping for the DQA1 or DQA4 allelic type is required, the signals on the membranes can be decolorized by washing in 0.18% Na_2SO_3 at room temperature with shaking; this can take from 5 minutes to 1 hour depending on signal intensity. The annealed probes are then stripped by incubating the membrane in water and 0.5% SDS at 65°C for at least 1 hour with shaking. The membranes can now be hybridized with subtyping probes.

Reverse Dot Blots

1. Pre-wet Genatran or ZetaProbe nylon membrane in water or 2× SSPE.
2. Spot 4 pmol each of oligonucleotide probe tailed with poly(dT) using terminal transferase (see Saiki *et al.* 1989 for details).
3. Fix the probe to the membrane by UV-cross linking at 50 mjules using Stratagene Stratalinker apparatus.
4. Incubate the membrane for at least 30 minutes at 55°C in 5× SSPE, 0.5% SDS to remove the unbound probe.
5. The membrane can be stored dry at room temperature for future use.

Hybridization

1. Pre-warm hybridization solution (5× SSPE, 0.5% SDS) to 55°C before use.

2. Denature biotinylated PCR product at 95°C for 5 minutes and place on ice.
3. Place the membrane in Seal-a-Meal bags or troughs, add 2 to 3 ml hybridization solution, 20 μl denatured PCR product, and 15 μl of 20 mg/ml HRP-SA stock solution. Remove air bubbles and seal the bag or cover trough tray with a glass plate and place a lead doughnut weight on top of the tray.
4. Hybridize for 20 minutes at 55°C in a shaking water bath.
5. Wash the membrane in a bowl containing 200 to 300 ml of prewarmed 2× SSPE, 0.1% SDS for 10 minutes in a shaking water bath at 55°C.
6. Proceed to the detection procedure.

Potential Problems

Contamination or carry-over from a previous PCR amplification can lead to problems in interpreting typing results. In general, this can be minimized by careful laboratory procedure (see Chapter 17) and monitored by the use of negative control (no template DNA) samples. In most cases of genetic typing, a contaminant can be detected as the presence of more than two alleles at a polymorphic locus.

For some loci, in particular at the HLA-DPβ locus (Bugawan *et al.* 1989), specific alleles cannot be defined by the hybridization of a particular oligonucleotide probe but by the pattern of probes that hybridize to the amplified DNA sample. The absence of HLA-DP allele-specific sequences and the requirement for multiple probes defining each individual allele reflects the patchwork pattern of polymorphism at this locus. Occasionally, an ambiguous typing result can be obtained when the sequence detected by a given probe could be assigned to either of the two alleles. In this case, the ambiguity can usually be resolved by using an allele-specific primer for amplification. This issue is discussed for HLA-DRβ typing with oligonucleotides in Scharf *et al.* (1989).

Another potential problem is that, on occasion, some nylon membranes appear to produce more "cross-hybridization" of the sequence-specific oligonucleotide typing probes, even when the hybridization and wash conditions remain constant. If this occurs,

some minor modification (e.g., increasing stringency by lowering the salt concentration or increasing the temperature) may be required to address the variability in membrane properties.

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